Potentiation of Insulin Signaling in Tissues of Zucker Obese Rats After Acute and Long-Term Treatment With PPARγ Agonists

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Thiazolidinediones (TZDs), agonists of peroxisome proliferator–activated receptor-γ (PPARγ), improve insulin sensitivity in vivo, and the mechanism remains largely unknown. In this study, we showed that, in Zucker obese (*fa/fa*) rats, acute (1-day) treatment with both rosiglitazone (a TZD) and a non-TZD PPARγ agonist (nTZD) reduced plasma free fatty acid and insulin levels and, concomitantly, potentiated insulin-stimulated Akt phosphorylation at threonine 308 (Akt-pT308) in adipose and muscle tissues. A similar effect on Akt was observed in liver after a 7-day treatment. The increase in Akt-pT308 was correlated with an increase in Akt phosphorylation at serine 473 (Akt-pS473), tyrosine phosphorylation of insulin receptor β subunit and insulin receptor substrate-1, and serine phosphorylation of glycogen synthase kinase-3α/β. The agonists appeared to potentiate Akt1 phosphorylation in muscle and liver and both Akt1 and Akt2 in adipose. Finally, potentiation of insulin signaling was also observed in isolated adipose tissue ex vivo and differentiated 3T3 L1 adipocytes in vitro, but not in rat primary hepatocytes in vitro. These results suggest that *1*) PPARγ agonists acutely potentiate insulin signaling in adipose and muscle tissues and such regulation may be physiologically relevant to insulin sensitization in vivo; *2*) the agonists directly target adipose tissues; and *3*) the metabolic and signaling effects of the agonists are mediated by structurally distinct PPARγ agonists. *Diabetes* 51:2412–2419, 2002

Thiazolidinediones (TZDs) (e.g., troglitazone, pioglitazone, and rosiglitazone) are a class of antidiabetic drugs that act as insulin sensitizers by decreasing insulin resistance in human and animal models (1,2). TZDs decrease circulating levels of insulin, free fatty acids, and triglycerides and increase insulin-stimulated glucose uptake and utilization (1,2). Peroxisome proliferator–activated receptor-γ (PPARγ) is a member of a larger family of the ligand-activated nuclear receptor transcription factors (3). In in vitro systems, TZD and select non-TZD antidiabetic compounds of different structural classes bind to PPARγ with high affinity and specificity, promote interaction of PPARγ with transcriptional coactivators or corepressors, increase PPARγ-mediated transcription regulation, and promote PPARγ-mediated cellular effects such as adipogenesis (3–5). Furthermore, in vivo efficacy in rodents generally correlates with in vitro PPARγ activity, suggesting that PPARγ activation is the predominant mechanism for the antidiabetic efficacy of the PPARγ agonists (3,6).

The mechanism by which activation of PPARγ leads to insulin sensitization is not fully understood. PPARγ and TZDs regulate the expression of several dozens of genes involved in a variety of cellular functions, including the metabolism of carbohydrates, fatty acids, triglycerides, and cholesterol (3,7,8). It is not clear which, if any, of these genes may be critical for insulin sensitization. It is also unknown which of the insulin-responsive tissues is the key target of TZDs, though adipose tissue is the leading candidate. PPARγ is expressed at high levels in the adipose tissue but at much lower levels in muscle and liver tissues (9,10). PPARγ agonists regulate adipocyte differentiation and metabolism (7,11). However, the use of genetic manipulation to produce mice with adipose tissue ablation yielded contradictory results on whether adipose tissues are required for the antidiabetic effects of TZDs (12,13). Finally, it remains possible, though unlikely, that TZDs may exert their major effects independently of PPARγ (14).

Activation of phosphatidylinositol 3-kinase (PI3K) is an early and key event in insulin signaling. PI3K activation results in rapid rise in phosphatidylinositol 3,4,5-trisphosphate (PIP3) that subsequently activates PIP3-dependent serine/threonine kinases, including Akt (15). Akt is known to undergo phosphorylation and activation upon insulin stimulation and is thought to play important roles in insulin-regulated metabolic effects (16). It was reported recently that genetic deletion of Akt2 but not Akt1 in mouse model is associated with insulin resistance and diabetes (17,18). Insulin-stimulated PI3K and Akt activation have been found to be blunted in the diabetic and insulin-resistant states in both animals and humans (19–28). It has been reported that TZDs exert their insulin-sensitizing effects at least partially by potentiating insulin-stimulated PI3K and Akt activation (27,29–34). However, it

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GSK, glycogen synthase kinase; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PPARγ, peroxisome proliferator–activated receptor-γ; nTZD, non-TZD PPARγ agonist; TZD, thiazolidinedione.

is not clear whether TZD-mediated effects on PI3K and Akt activation are an early event that contributes to insulin sensitization or a late event consequential to improved insulin sensitivity in vivo.

The current study used both cellular and animal models to investigate the effects of PPARγ agonists on multiple components of the insulin signaling pathway. The results showed that PPARγ agonists potentiated insulin signaling in vivo in a tissue- and time-dependent fashion, and that such potentiation may play a role in PPARγ agonist–mediated insulin sensitization in vivo.

RESEARCH DESIGN AND METHODS

Materials. Insulin was purchased from Sigma. Rabbit polyclonal antibodies against insulin receptor β-subunit and insulin receptor substrate-1 (IRS-1), sheep polyclonal antibodies against Akt1 and Akt2, and mouse monoclonal antibody 4G10 specifically against tyrosine phosphorylated proteins were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against Akt isoforms phosphorylated at a position equivalent to threonine 308 (Akt-pT308) and serine 473 (Akt-pS473) in Akt1, mouse monoclonal antibody against glycogen synthase kinase-3α/β ($GSK3_{\alpha/\beta}$), and polyclonal antibody against both $GSK3_{\alpha}$ phosphorylated at serine 21 and $GSK3_{\beta}$ phosphorylated at serine 9 ($GSK3_{\alpha/\beta}$-pS21/9) were purchased from Cell Signaling (Beverly, MA). The TZD rosiglitazone [(+/−)-5-(4-(2-(methyl-2-pyridinylamino)ethoxy)phenyl)methyl)-2,4-thiazolidinedione] was used in these studies. In addition, a novel indole-acetic acid PPARγ agonist, nTZD [2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid] was kindly provided by Drs. Derek Von Langen and Michael Kress of Merck Research Laboratories (Rahway, NJ).

Cell culture, isolation of primary hepatocytes, compound treatment, and protein extraction. 3T3 L1 cells were maintained and differentiated into adipocytes as previously described (35). Primary hepatocytes were isolated from male Sprague Dawley (SD) rats as previously described (36). 3T3-L1 adipocytes or rat primary hepatocytes were first incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) containing PPARγ agonists or vehicle (DMSO) for 16 h and then exposed to insulin for 15 min at 37°C. The cells were rinsed with ice-cold PBS, and then lyzed in ice-cold lysis buffer that contains 20 mmol/l HEPES pH 7.4, 1% Triton X-100, 20 mmol/l β-glycerophosphate, 150 mmol/l NaCl, 1 mmol/l sodium orthovanadate, 10 mmol/l sodium fluoride, and 1× concentration of a protease inhibitor cocktail (Roche Diagnostics, Germany). The cell lysates were cleared by centrifugation. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA).

In vivo experiments with genetically obese Zucker (*fa/fa*) rats. Lean and obese Zucker female rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were kept on a 12-h light/dark cycle at constant room temperature, and conventional laboratory diet and tap water were provided ad libitum until 4 h before euthanasia, when food was withdrawn. There were eight rats per treatment group. The rats were dosed with compounds at 30 mg/kg body wt or vehicle (0.5% methycellulose) via oral gavage in the morning for 1, 2, or 7 days. Blood samples were obtained from the tail vein immediately before dosing (predose) and 24 h after the last dose under the ad libitum–fed condition. The concentrations of insulin, free fatty acids, and triglycerides in the blood were measured as previously described (37). After anesthesia, ~200–300 mg liver, abdominal epididymal fat, and soleus muscle of one hindleg were removed from each rat. Insulin was then infused via portal veins at the dose of either 0.5 or 5 units/kg body wt. A similar amount of liver, abdominal epididymal fat, and soleus muscle of the other hindleg was removed from the rat 30, 60 and 120 s after insulin infusion, respectively. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Proteins were extracted from frozen tissue samples at 4°C in the lysis buffer described above with the aid of a Polytron homogenizer (Fisher, Pittsburgh, PA). The method for removing the tissue samples from intact animals before and after insulin treatment has been used in earlier publications (38).

Ex vivo treatment of adipose tissue. Abdominal epididymal fat was obtained from obese Zucker female rats and cut into small pieces in Media 199 (Life Technologies, Grand Island, NY) adjusted to pH 7.2 with HEPES. After brief centrifugation, the adipose tissue (at top) was removed and resuspended in fresh Medium 199 containing vehicle (DMSO), rosiglitazone, or nTZD at 37°C and 10% CO_2 for 5 h with gentle shaking. The adipose tissues were then exposed to vehicle (0.1N acetic acid) or insulin for 15 min, separated from medium by centrifugation, and then lyzed as described above for frozen tissues.

Immunoprecipitation and Western blot analysis. For immunoprecipitation, lysates were mixed with antisera for 2–4 h and then protein-A or protein-G agarose beads for another 1–4 h at 4°C with gentle shaking. The beads were washed with lysis buffers for three times. Cell and tissue lysates or immunoprecipitations were resuspended in SDS-loading buffer (Invitrogen, Carlsbad, CA) and separated in precast 4–20% gradient NuPAGE SDS-PAGE gels (Invitrogen, Carlsbad, CA). The proteins were then transferred to a polyvinylidine fluoride (PVDF) membrane and probed with primary antibody. Detection was performed with Phototope-HRP Western blot Detection Kit by film exposure (New England BioLabs, Beverly, MA) or ECF Western Blotting Kit (Amersham Pharmacia Biotech, Piscataway, NJ) by scanning with a Storm gel and blot imaging system (Molecular Dynamics), per the manufacturer's recommendation.

Akt1 and Akt2 were isolated from tissue lysates with equivalent amounts of total proteins by immunoprecipitation with Akt1- and Akt2-specific sheep polyclonal antibody, respectively. The levels of total or phosphorylated Akt1 and Akt2 in the immunoprecipitates were determined by Western blot using the antibody against all isoforms of total Akt or Akt-p308, respectively. The specificity of the isoform-specific antibodies was confirmed by experiments showing, for example, that Akt2 antibody but not Akt1 antibodies detected a 60-kDa band in the immunoprecipitation of Akt2 antibody from adipose tissue lysate (data not shown). An excessive amount (20 μg) of Akt1 and Akt2 antibodies was used in each of the immunoprecipitates. That Akt1 and Akt2 proteins were completely immunoprecipitated from the tissue lysates was confirmed by experiments showing that Akt1 and Akt2 antibodies clearly detected an ~60-kDa band in the lysate before but not after the corresponding immunoprecipitates (data not shown).

For determination of tyrosine phosphorylation of IRS-1 and insulin receptor, IRS-1 and insulin receptor β subunit were isolated from adipose and muscle tissue lysates with equivalent amounts of total proteins (1 mg) by immunoprecipitation with rabbit polyclonal antibody against IRS-1 and insulin receptor β subunit, respectively. The levels of total or tyrosine-phosphorylated IRS-1 and insulin receptor β subunit in the immunoprecipitates were determined by Western blot using rabbit polyclonal antibody against IRS-1, rabbit polyclonal antibody against insulin receptor β subunit, and the mouse monoclonal antibody 4G10 against tyrosine-phosphorylated proteins, respectively. For determination of phosphorylated $GSK3_{\alpha}$ and $GSK3_{\beta}$, Western blots were performed using crude tissue lysates with equivalent amounts of total proteins (50 μg) and antibodies against total or phosphorylated $GSK3_{\alpha/\beta}$ proteins.

Statistics. All data are presented as means + SE. Statistical significance was determined by unpaired Student's t test. $P < 0.05$ was considered significant (marked with *), and $P < 0.01$ was considered very significant (marked with **).

RESULTS

Rosiglitazone and nTZD acutely reduce circulating insulin and free fatty acid levels in Zucker obese rats. We investigated the physiological effects of PPARγ agonists using genetically obese Zucker female rats and their lean littermates as controls. The obese rats were dosed with vehicle, rosiglitazone, or nTZD for 1, 2, and 7 days. As expected, Zucker obese rats manifested hyperinsulinemia, with plasma insulin concentrations >20 times (~12 ng/ml) those of lean rats (~0.5 ng/ml) (Fig. 1*A*). In the obese rats, rosiglitazone and nTZD treatment significantly reduced plasma insulin (Fig. 1*A*) and free fatty acid levels (Fig. 1*C*) within the first day of treatment and reduced plasma triglyceride levels within the first 2 days of treatment (Fig. 1*B*). Taken together, these results demonstrate that PPARγ agonist treatment improved in vivo metabolic profiles of Zucker obese rats. In particular, circulating insulin and free fatty acid levels were acutely reduced within the first day of treatment.

PPARγ agonists potentiate insulin signaling in adipose and muscle acutely but in liver only after longer-term treatment in Zucker obese rats. To determine the effects of PPARγ agonists on insulin signaling in vivo, lean rats and vehicle or PPARγ agonist–treated obese rats were

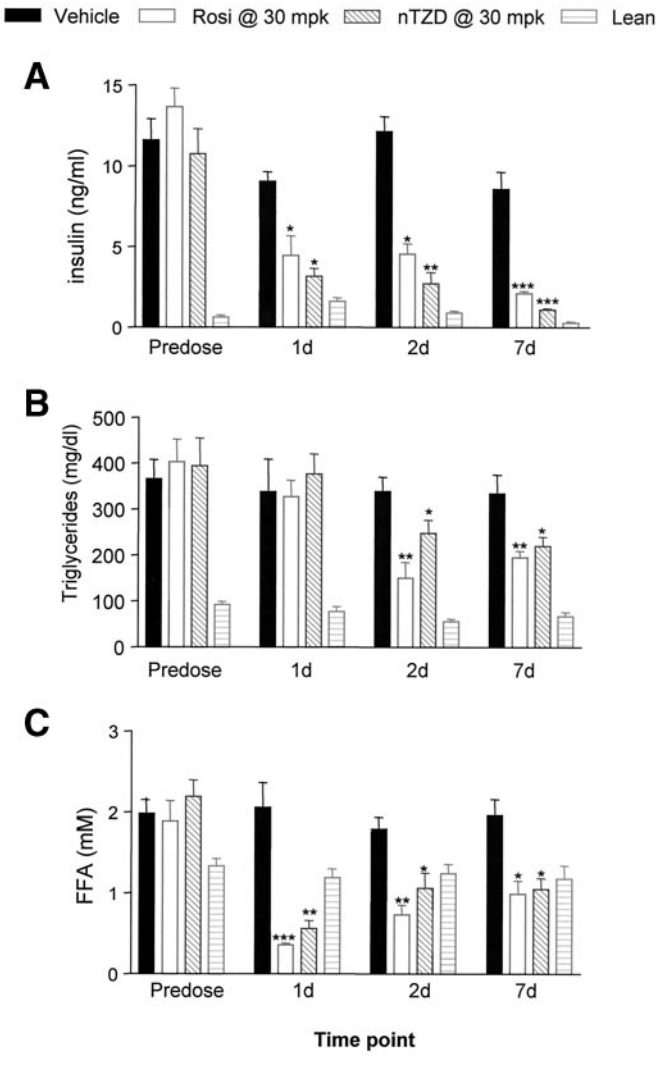

FIG. 1. Time course of the effects of short-term rosiglitazone treatment on metabolic parameters in Zucker obese and lean female rats in vivo. Zucker obese rats were treated with vehicle, rosiglitazone (Rosi), or nTZD at 30 mg/kg for 1 day, 2 days, and 7 days. Age-matched lean rats were used as controls. Each treatment group had eight rats. *A*: Circulating insulin concentration. *B*: Circulating triglyceride concentration. *C*: Circulating free fatty acid concentration. The error bars represent standard error of the means, and an unpaired *t* test was used for comparison. *$P < 0.05$, vehicle vs. compound-treated groups. mpk, milligrams per kilogram body weight.

subjected to insulin infusion. Abdominal epididymal fat, hindleg soleus muscle, and liver samples were obtained from the rats immediately before and after insulin infusion. Components of insulin signaling cascades in these tissues were then examined by Western blot analysis. Two in vivo experiments were performed in the current studies.

In the first experiment, obese rats were treated with vehicle or rosiglitazone at 30 mg/kg body wt and insulin was perfused at 0.5 units/kg body wt. As shown in Fig. 2*A*, in the fat, the levels of basal Akt phosphorylation at threonine 308 (Akt-pT308) were comparable among all the different groups of rats before insulin infusion. Upon insulin infusion, Akt-pT308 was significantly induced in the fat of the lean rats and in 1- and 2-day rosiglitazone–treated obese rats, but not in vehicle-treated obese rats. Similar effects were observed on Akt-pS473 (Fig. 2*E*), $GSK3_{\alpha/\beta}$-pS21/9 (Fig. 2*F*), and tyrosine phosphorylation of insulin receptor β subunit (Fig. 3*A*) and IRS-1 (Fig. 3*B*). Taken together, these results suggest that insulin signaling is impaired in the adipose tissues of the obese rats and that such impairment can be acutely improved by rosiglitazone treatments.

As shown in Fig. 2*B*, while insulin-stimulated Akt-pT308 were observed in the hindleg soleus muscle of all groups of rats, the induction was the lowest in the vehicle-treated obese rats. Furthermore, while insulin-stimulated tyrosine phosphorylation of insulin receptor β subunit was comparable among the different groups of rats (Fig. 3*C*), insulin-stimulated tyrosine phosphorylation of IRS-1 was the lowest in the vehicle-treated obese rats (Fig. 3*D*). Taken together, these results suggest that insulin signaling in the muscle tissues of the obese rats was at least partially impaired and that such impairment, as in the adipose tissues, was improved by rosiglitazone within the first day of treatment.

In the experiment described above, insulin signaling was not detected in liver tissue (data not shown). Therefore, we performed a second in vivo experiment in which the rats were infused with a higher level (5 units/kg body wt) of insulin. In this second experiment, the rats were treated with rosiglitazone as well as nTZD and for 1, 2, and 7 days. At this level of insulin, comparable stimulation in Akt phosphorylation was observed in all groups of rats in both fat (Fig. 2*C*) and muscle (data not shown), indicating that insulin response was maximally stimulated. Therefore, the effects of rosiglitazone and nTZD on insulin signaling in fat and muscle cannot be determined in this experiment. In liver, however, the response to 5 units/kg body wt insulin was graded (Fig. 2*D*). In comparison with vehicle-treated obese rats, significantly higher induction of Akt-pT308 was observed in the 7-day but not in the 1-day (data not shown) and 2-day rosiglitazone– or nTZD-treated obese rats. Taken together, these results suggest that the effects of PPARγ agonists on insulin signaling in liver tissue required longer-term (>2 days) treatment.

While rosiglitazone and/or nTZD potentiated insulin signaling in all the tissues examined, the agonists did not increase basal insulin signaling in any of the tissues. In fact, it appeared to decrease at least basal Akt phosphorylation in muscle (Fig. 2*B*) and in liver (Fig. 2*D*) after 2 days of treatment.

PPARγ agonists potentiate insulin-stimulated Akt1 phosphorylation in muscle and liver and both Akt1 and Akt2 in adipose. We investigated whether PPARγ agonists differentially affect Akt1 and Akt2 activation in vivo. Akt3 was not characterized due to the lack of suitable antibodies for immunoprecipitations and Western blots. As shown in Fig. 4*A*, in adipose tissues, Akt1 protein was found to be at apparently higher levels than total Akt2 protein (top panel). Furthermore, upon insulin stimulation, both phosphorylated Akt1 and Akt2 were clearly detectable in the 1-day rosiglitazone–treated rats but not in vehicle-treated rats (bottom panel). As shown in Fig. 4*B*, in muscle, Akt1 protein was also found to be at higher levels than Akt2 protein (top panel). Upon insulin infusion, Akt1 phosphorylation in vehicle-treated rats was detectable but apparently at lower levels than in 1-day rosiglitazone–treated rats (bottom panel). Similarly low levels of Akt2

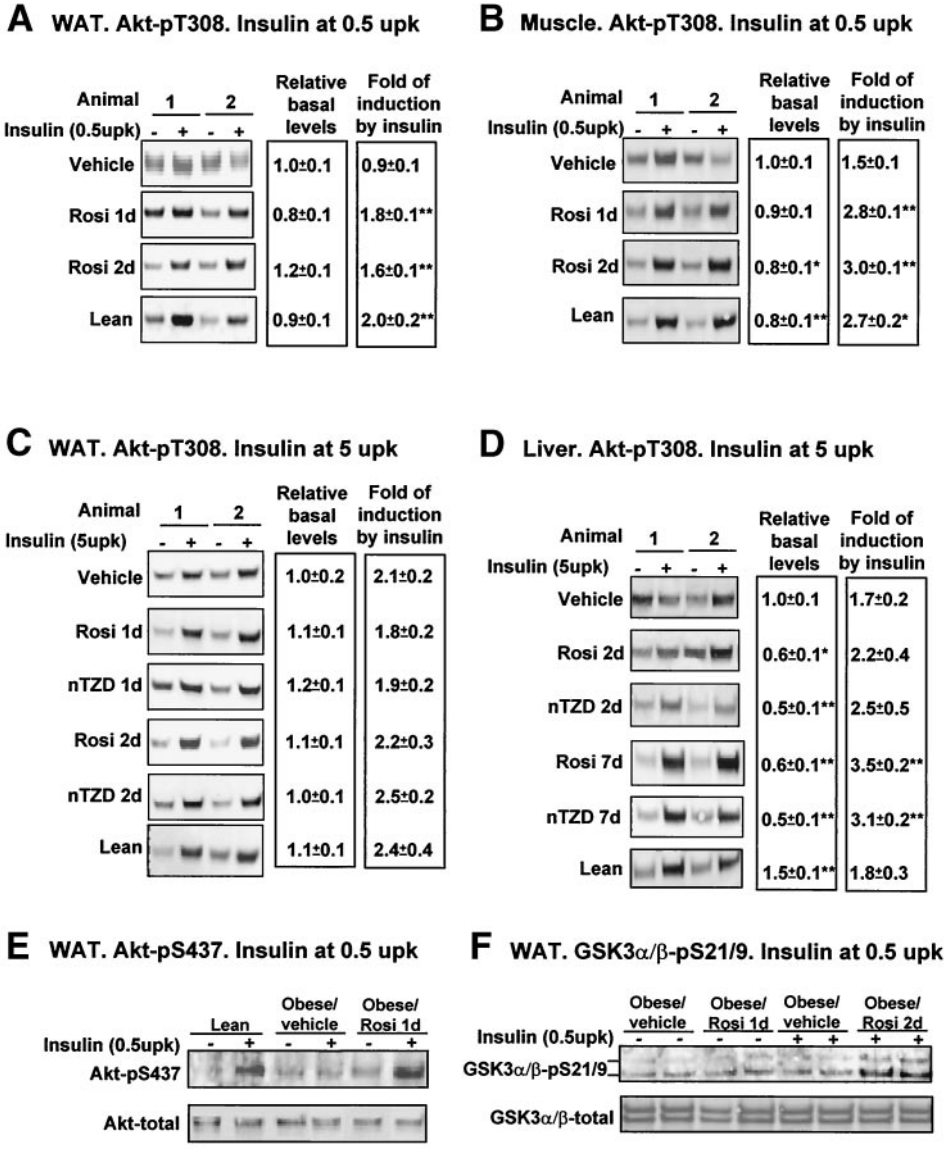

FIG. 2. Time course of the effects of short-term rosiglitazone treatment on insulin signaling in Zucker obese and lean female rats in vivo. Equal amounts of proteins extracted from rat tissues were loaded into each lane. For panels *A*, *B*, *C*, *D*, and *E*, total Akt or Akt phosphorylated at position equivalent to threonine 308 in Akt1 (Akt-pT308) or serine 473 in Akt1 (Akt-pS473) were determined by Western blots as described in RESEARCH DESIGN AND METHODS. All quantification was based on a total of eight rats per treatment. Only two (2) rats per treatment are pictured in the blots. For panel *F*, Western blots for $GSK3_{\alpha}$ and $GSK3_{\beta}$ phosphorylated at serine 21 and 9 ($GSK3_{\alpha/\beta}$-pS21/9) or both phosphorylated and unphosphorylated $GSK3_{\alpha}$ and $GSK3_{\beta}$ (total $GSK3_{\alpha/\beta}$). For panels *A*, *B*, *E*, and *F*, the rats were dosed with insulin at 0.5 units/kg body wt (upk). For panels *C* and *D*, the rats were dosed with insulin at 5 units/kg body wt. The average level of phosphorylated Akt in vehicle-treated obese rats was set to unit (1). Fold induction was calculated for each of the rats and averaged across the eight rats within a treatment group.

phosphorylation were detected in both rosiglitazone- and vehicle-treated rats (bottom panel).

As shown in Fig. 4*C*, in liver, Akt1 protein was also found to be at higher levels than Akt2 protein (top panel). Upon insulin infusion, Akt1 phosphorylation in vehicle-treated rats was detectable but at apparently low levels than in either 7-day rosiglitazone– or nTZD-treated rats (bottom panel). Similarly low levels of Akt2 phosphorylation were detected in all groups of rats (bottom panel). Taken together, these results indicate that PPARγ agonists potentiate insulin-stimulated phosphorylation of Akt1 in adipose, muscle, and liver and both Akt1 and Akt2 in adipose.

Rosiglitazone and nTZD potentiate insulin signaling in isolated adipose tissue ex vivo. We investigated whether PPARγ agonists could directly target the adipose tissues. Abdominal epididymal fat was isolated from Zucker obese rats, pretreated with vehicle (DMSO), rosiglitazone, or nTZD for 5 h and then subjected to insulin stimulation for 15 min. As shown in Fig. 5, Akt phosphorylation was not detectable either at the basal state or upon 1 nmol/l insulin stimulation (top panel *lanes 1* and *2*), but was clearly detectable upon 100 nmol/l insulin stimulation (top panel *lane 3*). Rosiglitazone and nTZD alone did not induce Akt phosphorylation (top panel *lanes 4* and *5*). While the agonists did not affect Akt phosphorylation upon 1 nmol/l insulin (top panel *lanes 6* and *7* vs. *lane 2*), they significantly increased Akt phosphorylation at 100 nmol/l insulin (top panel *lanes 8* and *9* vs. *lane 3*). No changes were detected for the levels of total Akt (bottom panel).

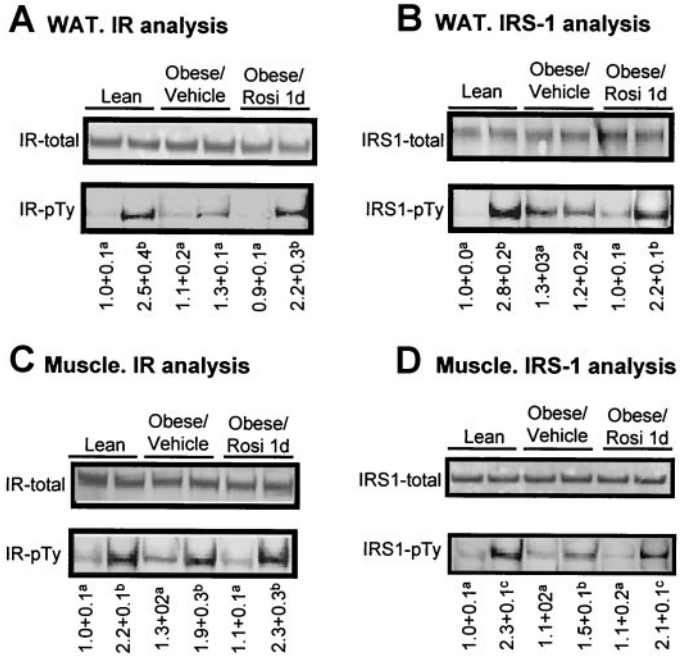

FIG. 3. Effects of TZDs on insulin-stimulated tyrosine phosphorylation of insulin receptor (IR) and insulin receptor substrate one (IRS-1) in Zucker obese rats in vivo –Shown are Western blots for total and tyrosine phosphorylated IR and IRS-1 in adipose and muscle using lysates pooled from all eight (8) rats. Quantification was based on additional Western blots (not shown) on the four individual rats of each of the treatments. Treatments that are different statistically ($P < 0.05$) were marked with different letters.

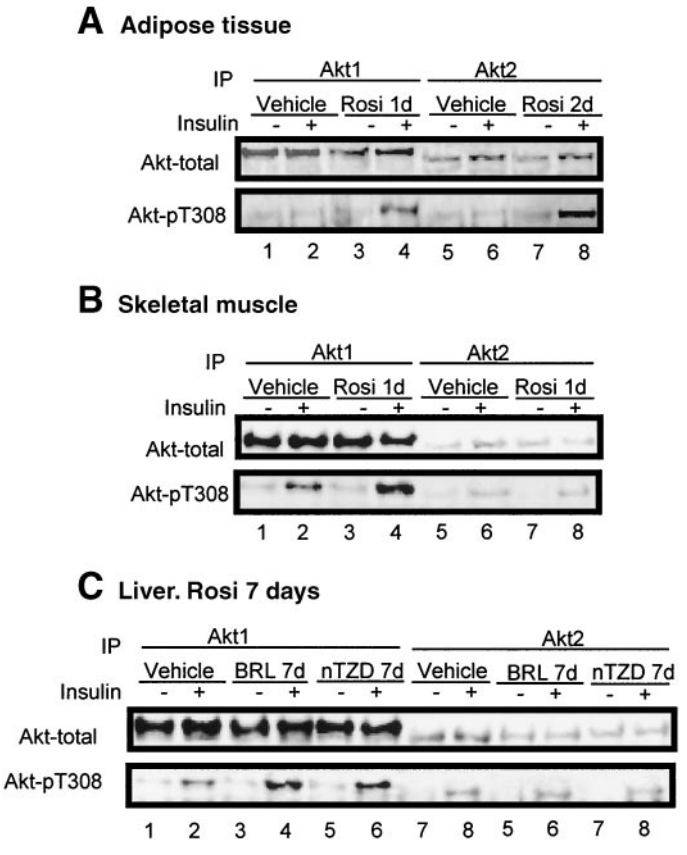

FIG. 4. Effects of TZDs on insulin-stimulated phosphorylation of Akt1 and Akt2 in Zucker obese rats in vivo. Lysates of adipose (*A*), muscle (*B*), and liver tissues (*C*) were pooled from all eight rats (8). Akt1 and Akt2 proteins were isolated with isoform-specific Akt antibodies by immunoprecipitation. The levels of total and phosphorylated Akt1 and Akt2 were then determined by Western blotting as described in RESEARCH DESIGN AND METHODS. Shown are the results of Western blots.

PPARγ agonists potentiate insulin signaling in differentiated 3T3 L1 adipocytes but not rat primary hepatocytes in vitro. In addition to the in vivo and ex vivo experiments described above, we also determined the effect of PPARγ agonists on insulin signaling in both differentiated 3T3 L1 adipocytes and rat primary hepatocytes in vitro. As shown in Fig. 6*A*, in differentiated 3T3 L1 adipocytes, insulin stimulation resulted in Akt phosphorylation in a dose-dependent fashion (top panel *lanes 1–4*). While rosiglitazone alone resulted in only slight (almost nondetectable) Akt phosphorylation without insulin stimulation (top panel *lanes 5* and *6*), it significantly potentiated phosphorylation of Akt stimulated by both 1 nmol/l (top panel *lane 7* vs. *lane 3*) and 10 nmol/l insulin (top panel *lane 8* vs. *lane 4*). The effects were not due to changes in the levels of total Akt protein (bottom panel *lanes 1–8*). Similar effects were also observed for nTZD on 3T3 L1 adipocytes (Fig. 6*B*). On the other hand, in primary rat hepatocytes, rosiglitazone did not affect phosphorylation of Akt stimulated by either 1 nmol/l (Fig. 6*C*, top panel *lane 2* vs. *lane 5*) or 10 nmol/l insulin (*lane 3* vs. *lane 6*).

DISCUSSION

Several studies have suggested that insulin-stimulated activation of PI3K and Akt is impaired in insulin-resistant cells, animals, and humans (19–23,25,26,28). Furthermore, such impairment can be improved by TZD treatments (27,29, 30,32–34). The current study used in vitro, ex vivo, and in vivo approaches to examine the effects of TZD and non-TZD PPARγ agonists on insulin signaling after short- and long-term treatment. The study provided several observations. First, there was a severe reduction in insulin-stimulated but not basal Akt phosphorylation in insulin-responsive tissues in Zucker obese rats. Second, concomitant with their ability to significantly reduce serum free fatty acid and insulin levels, both rosiglitazone and a non-TZD PPARγ agonist acutely potentiated insulin signaling in adipose and muscle tissues within the first day

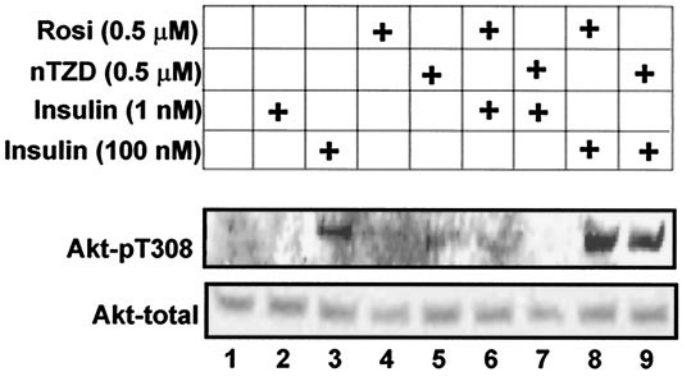

FIG. 5. Effects of rosiglitazone (Rosi) or nTZD on insulin-stimulated Akt phosphorylation in adipose tissue ex vivo. Abdominal adipose tissues from Zucker obese rats were minced and incubated with Rosi or nTZD for 5 h, exposed to insulin for 15 min, and then lysed. Equal amounts of proteins were loaded onto each lane. Shown are Western blots to detect both the level of Akt-pT308 (top panel) and total Akt (bottom panel). Similar results were obtained from three independent experiments.

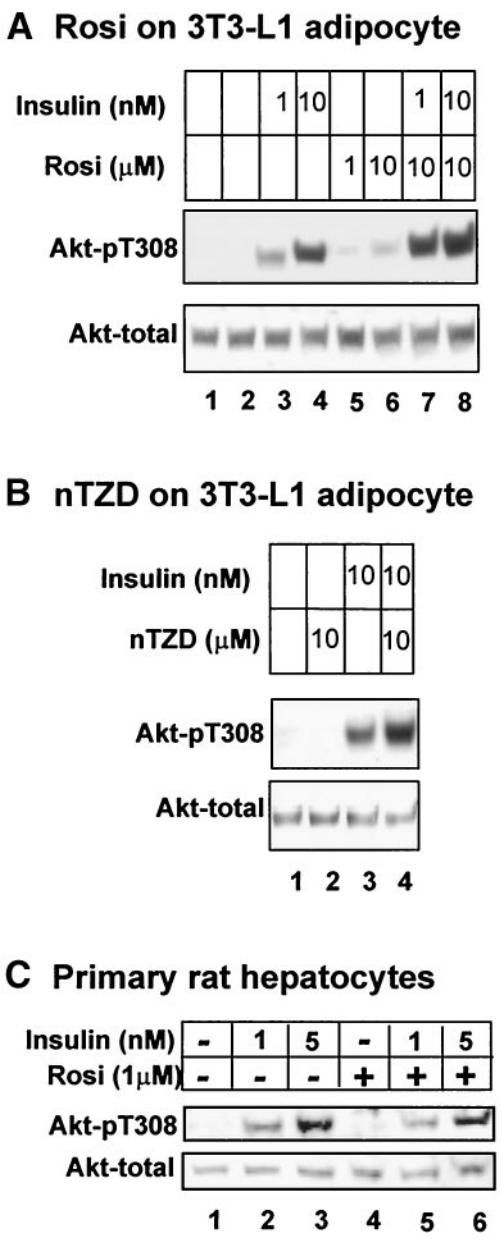

FIG. 6. Effects of rosiglitazone (Rosi) or nTZD on insulin-stimulated Akt phosphorylation in 3T3 L1 adipocytes and rat primary hepatocytes in vitro. Differentiated 3T3-L1 adipocytes (*A* and *B*) and primary rat hepatocytes (*C*) were cultured, serum starved, and incubated with rosiglitazone or nTZD (10 μmol/l) overnight. The cells were then exposed to insulin and lysed in lysis buffer. Shown are the results of Western blots. For each part of the figure, similar results were obtained in at least two independent experiments.

of treatment. On the other hand, potentiation on Akt phosphorylation in liver tissues required longer treatment. Third, rosiglitazone potentiates Akt1 in muscle and liver tissues and both Akt1 and Akt2 in adipose. Finally, both rosiglitazone and the non-TZD PPARγ agonist acutely potentiate insulin signaling in isolated adipose tissues ex vivo in differentiated 3T3 L1 adipocytes but not in rat primary hepatocytes in vitro.

Although it is generally thought that TZDs mediate their insulin-sensitizing effects via PPARγ, PPARγ independent pathways have also been suggested. For instance, it has been proposed that the acute effects of TZDs observed in vivo are too rapid to be accounted for by transcriptional regulation (14,39,40). Such proposals are particularly relevant given the recent finding that, in PPARγ-deficient embryonic stem cells, TZDs have anti-inflammatory effects (41). In this study, structurally distinct rosiglitazone and non-TZD PPARγ agonists potentiate insulin signaling. This is consistent with the notion that the effects were mediated directly via PPARγ instead of unknown off-target activity.

Long-term TZD treatment has been shown to activate PI3K and/or Akt in vivo (27). Long-term TZD treatment generally results in significant changes in multiple metabolic parameters, including the levels of circulating glucose, free fatty acids, and insulin. It is unclear whether the improved insulin signaling leads to improved metabolic parameters or visa versa. By analyzing multiple components of the insulin signaling pathway (i.e., insulin receptor, insulin receptor substrate, Akt, and GSK3), the current study demonstrated that both rosiglitazone and nTZD PPARγ agonists acutely potentiated insulin signaling in both the adipose (Fig. 2*A*) and the muscle tissues (Fig. 2*B*) within the first day of treatment. These results suggest that such potentiation is an early and probably primary effect. It has been proposed that TZDs improve insulin sensitivity by affecting adipose cell differentiation (42). The results from the current study suggest that the effects on differentiation may be relevant for the long-term therapeutic effects of TZDs but are unlikely to be critical for the insulin sensitization, at least in short-term treatments.

The identity of the physiologically relevant target tissues of TZDs or other PPARγ agonists in vivo remains controversial. While muscle and liver tissues account for most glucose disposal and production in vivo, PPARγ is expressed at much lower levels in these tissues than in the adipose tissues. However, recent genetic studies have provided contradictory results on the role of adipose tissues in the antidiabetic function of TZDs (12,13). The current study provides several lines of evidence supporting direct effects of PPARγ agonists on adipose tissues. First, potentiation of insulin signaling in adipose tissues was observed within the first day of treatment with PPARγ agonists (Figs. 2 and 3). Second, PPARγ agonists acutely potentiated insulin signaling in isolated adipose tissues ex vivo (Fig. 5) and differentiated 3T3 L1 cells in vitro (Fig. 5). The current study also provides evidence supporting indirect effects of PPARγ agonists on liver tissues. First, PPARγ agonists altered metabolic parameters acutely (Fig. 1) but potentiated insulin signaling in liver tissues only in longer-term treatment (Fig. 2*D*). Second, rosiglitazone potentiates insulin signaling in 3T3 L1 adipocytes but not rat primary hepatocytes (Fig. 6).

On the other hand, we are uncertain about whether PPARγ agonists target the muscle tissues in direct or indirect fashions. While the result that PPARγ agonists acutely potentiated insulin signaling within the first day of treatment (Fig. 2*B*) suggests an early and, therefore, probably primary effect, our previous study showed that ex vivo incubation of mouse soleus muscle strips with PPARγ agonists did not result in improvement of insulin-stimulated glucose uptake (10). Furthermore, it was reported that TZD-mediated insulin-sensitizing effects are intact in muscle-specific PPARγ knockout mice (43).

Therefore, further studies are needed to address the effects of PPARγ agonists on muscle.

The acute potentiation of insulin signaling in adipose and muscle (Fig. 2*A* and *B*) occurred concomitantly with improved metabolic profiles, including reduced insulin and free fatty acid levels in the plasma (Fig. 1), suggesting that the signaling effects are physiologically relevant to PPARγ agonist–mediated antidiabetic effects in vivo. Decreased circulation free fatty acids levels may contribute to improve insulin sensitivity in tissues (44). It was reported recently that Akt2 but not Akt1 deficiency in mice is associated with insulin resistance and diabetes, which supports the notion that Akt2 is important in insulin action (17,18). In the current study, we observed that PPARγ agonists appear to potentiate Akt1 but not Akt2 in muscle and liver (Fig. 4*B* and *C*). On the other hand, it appears that both Akt1 and Akt2 were potentiated in the adipose tissue (Fig. 4*C*). The potentiation of Akt2 activation in the adipose tissues may therefore be of particular physiological relevance. Based on the current study alone, however, we cannot exclude the role of Akt1 potentiation in insulin sensitization in adipose and other tissues.

Our in vivo results showed that PPARγ agonists significantly reduced rather than increased the basal Akt phosphorylation in both muscle (Fig. 2*B*) and liver tissues (Fig. 2*C*). The observation that TZD and non-TZD PPARγ agonists potentiate insulin action but do not activate the insulin signaling pathway by themselves in vivo may have important implications for PPARγ agonists as long-term therapeutics. It has been shown that overexpression of a membrane-targeted PI3K p100 catalytic subunit resulted in constitutive activation of Akt and subsequent insulin resistance (45,46). It is therefore possible that, by not constitutively activating the insulin signaling pathway, PPARγ agonists are less likely to cause insulin resistance in long-term treatment.

In conclusion, the current study suggests that PPARγ agonists potentiate insulin signaling in vivo in a tissue- and time-dependent fashion, and that such potentiation may play a role in PPARγ agonist–mediated insulin sensitization in vivo. The mechanisms by which PPARγ agonists potentiate insulin signaling warrants further investigation.

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